

IN THE SPECIFICATION:

On page 8, lines 1-14, please replace with the following paragraph:

As indicated above, loading of exosomes derived from dendritic cells (dexosomes) with HLA class I or II associated peptides has up to now been approached by indirect loading, i.e., by adding a therapeutic (antigenic) peptide (e.g., class I restricted tumor peptide) (e.g., at 10 μ g/ml on day 6) to the culture of vesicle producing cells (e.g., dendritic cells (DC) differentiated from monocyte-enriched adherent cells in the presence of GM-CSF and IL-4) (see for instance US5,846,827 and cited references). It is assumed that the DC take up the exogenous peptide and thereby produce dexosomes harboring these class I peptides on the exosome surface. *In vivo* experiments with the murine P1A mastocytoma tumor model show that dexosomes produced in this manner elicit anti-tumor immunological responses that resulted in tumor regression. Dexosomes indirectly loaded with Mart-1 (SEQ ID NO. 3) peptide could also stimulate marginal secretion of IFN- γ from a Mart-1 specific CTL clone, LT 11, suggesting that some degree of peptide loading did occur. However, even using time resolved fluorometry (TRF), a biochemical approach with sensitivity similar to radioactive detection, binding of class I peptide on these dexosomes was hardly detectable.

On page 27, lines 15-18, please replace with the following paragraph:

Figure 20. β 2-microglobulin facilitates direct peptide loading. Biotinylated reference peptide SEQ ID NO. 1) was loaded with β 2-microglobulin concentrations from 0-80 μ g/ml. The amount of peptide bound is indicated on the y-axis in terms of Eu fluorescence (counts per second) as a function of the β 2-microglobulin concentration.

On page 29, line 14 through page 30 line 5, please replace with the following paragraph:

Figure 30. IFN- γ secretion by LT 11 stimulated by dexosome loaded with Mart-1 peptide (SEQ ID NO. 3) in the absence of beta 2 microglobulin. LT 11 cells were stimulated by Mart-1 peptide (SEQ ID NO. 3) loaded dexosomes in the presence of DC as accessory cells and IFN- γ secretion from LT 11 is indicated on the y-axis as a function of dexosomes. As control, Exo 447 was also loaded at pH 4.2 in the presence of beta 2 microglobulin.

Figure 31. Binding (top panel) and inhibition (bottom panel) of HLA-A1 restricted peptide in the presence of beta 2 microglobulin. Top panel: HLA-A1 restricted, biotin-labeled peptide MAGE-3C5 (SEQ ID NO. 5) specifically bind to HLA-A1⁺ dexosomes. Bottom panel: the binding is inhibited by unlabeled MAGE-3A1 peptide (SEQ ID NO. 2).

Figure 32. Competition between HLA-A1/B35 restricted, biotin-labeled MAGE-3C5 (SEQ ID NO. 5) and unlabeled MAGE-3A1/B35 peptide on HLA-B35⁺ dexosomes (Exo 426) in the absence of beta 2 microglobulin.

On page 82, line 14 through page 83, line 12, please replace with the following paragraph:

12.1. Methods

Direct loading peptide to exosome in the presence and absence of β 2-m

HLA-A2 restricted reference peptide FLPSDCFPSV (SEQ ID NO. 1), derived from a natural epitope of Hepatitis B core antigen, is biotinylated via cysteine and competes with unlabeled reference peptide, suggesting that the biotinylated peptide maintains its ability to bind to HLA-A2 molecules. For direct peptide loading in the presence of β 2-m, 100 μ l of purified HLA-A2⁺ and HLA-A2⁻ negative

dexosomes are treated with an equal volume of citric acid or acetate buffer, pH 3.2-5.2 at 4°C for 90 seconds, lowering the pH to favor elution of bound endogenous peptides. After the treatment, the preparation is immediately neutralized with a cocktail of pH 11 containing Tris, exogenous peptide (from 0.01 to 10 μ g/ml final concentration) and α 2-m (from 0-80 μ g/ml final concentration), and incubated at room temperature to allow reformation of the tri-molecule complex of class I, α 2-m, and peptide on the dexosome surface. For direct loading in the absence of exogenous α 2-m, dexosomes are mixed with exogenous peptide (10 or 100 μ g/ml final concentration) first then with an equal volume of acetate buffer, pH 4.2-5.2 and incubated at room temperature for 30 minutes. During this period, the exchange of peptides occurs resulting in the binding of the exogenous peptide present in excess. The exogenous peptide can be biotinylated peptide alone or a mixture of the labeled peptide with increasing amount of a target peptide. Removal of unbound peptide and α 2-m is not necessary since the signal source is from Class I/peptide complexes captured by anti-HLA-Class I antibodies described as below.

On page 85, line 1 through page 86, line 2, please replace with the following paragraph:

Mart-1 specific CTL clone stimulation assay

Dexosomes directly loaded with MART-1 peptide (SEQ ID NO. 3) as described above are washed of free peptide after the loading, and incubated with DC and T cell clone LT 11 cells for 24 hours. Biological activity is measured by IL-2 secretion with an ELISA according to the following T cell clone assay.

The peptide used is an HLA-A2 restricted MART-1 peptide (SEQ ID NO. 3) having the sequence ELAGIGILTV.

Unbound Mart-1 peptide (SEQ ID NO. 3) is removed by density gradient centrifugation. It should be understood that the removal of unbound peptide from dexosome is not mandatory, but reduces unspecific binding.

T Cell Clone Assay

20x 10³ HLA A2 positive or negative monocyte derived dendritic cells (BM-DC) were incubated for 2 hours with 15 µl (1x10¹⁰ class ii) of the dexosome preparation in U-bottom 96-well plate, at 37°C, then, 20x 10³ cells of the T cell clone LT11 were added to the Human BM-DC cells. The final volume per well was of 200µl. 24 hours later, supernatants were harvested and analysed for IL-2 presence by ELISA. HLA A2⁺ BM-DC cells pulsed directly with 10µm of mart peptide (ELAGIGILTV) (SEQ ID NO. 3) were used as positive control.

P1A Peptide Loaded Dexosomes.

Murine H2^b and H2^d dexosomes were directly loaded with the OVA peptide 257-269 (SIINFEKL) (SEQ ID NO. 4) as follows : 1 volume of Na acetate buffer (0.2M, pH5) was added to 1 volume of exosomes and incubated, for 90 seconds, at 4°C, then neutralized by addition of tris 2M solution (pH 11) containing 10 µm of OVA peptide (SEQ ID NO. 4) and 40 µg/ml of human b2-microglobulin. After an incubation of 4h at room temperature OVA dexosome complexes were separated from unbound OVA peptide by an optiprep gradient.

On page 86, lines 9 through page 87, line 12, please replace with the following paragraph:

12.2. Results

Direct loading of HLA-A2⁺ dexosome in the presence of α 2-m

The direct peptide loading was first performed with HLA-A2 restricted reference peptide H-FLPSDC(biotin)FPSV-OH (SEQ ID NO. 1) on HLA-A2⁺ dexosomes, as described. HLA-A2⁻ dexosomes were used as HLA specificity control. Figure 17 shows HLA-A2 molecules loaded with biotin-labeled reference peptide captured on the plate by anti-Class I antibody after dexosome lysis. The binding of reference peptide to dexosome is HLA-A2 specific because it does not bind to HLA-A2⁻ dexosomes. The binding depends on both peptide and the amount of dexosome.

Parameters for direct peptide loading were assessed using the TRF assay described. Buffer choice was influenced by the effect on peptide loading efficiency and on dexosome integrity as measured by SEE assay. A range of pH of the loading buffers was tested to optimize conditions for loading of reference peptide. We found comparable signals for peptide binding were obtained using similar loading conditions with either citric acid phosphate buffer or sodium acetate buffer (Figure 18). To test whether mild acid treatment might change conformation of the molecules expressed on dexosome and impair their functions, we performed a superantigen assay in which superantigen SEE, bound to HLA-DR molecules of dexosomes, induced IL-2 secretion from Jurkat cells in the presence of Raji cells as accessory cells. Figure 19 shows that dexosome preparations treated with sodium acetate at pH 4.2 induced the same amount of IL-2 secretion as untreated control in the SEE assay. Dexosomes treated with citric acid/phosphate buffer at the same pH 4.2 also induced IL-2, although to a much lower extent. In this regard, citrate buffer may inactivate the exosomes so that conditions used to load whole cells using citrate buffer are not efficient for loading exosomes. The sodium acetate buffer was thus selected for further studies. We next tested whether the binding of peptide to dexosome HLA-A2 is enhanced by the presence of exogenous α 2-m. Figure 20 shows that with 10 μ g/ml of the HLA-A2

restricted biotin reference peptide (SEQ ID NO. 1), addition of 20 μ g/ml of α 2-m significantly increases the amount of the reference peptide bound.

On page 88, line 9 through page 89, line 15, please replace with the following paragraphs:

A functional assay using a MART-1 peptide specific T cell clone, LT11, was established to test the biological activity of dexosomes, which measures antigen specific T cell responses *in vitro*. Following the protocol for direct loading of dexosomes with class I peptides, a MART-1, HLA-A2 restricted peptide (SEQ ID NO. 3) was loaded onto HLA-A2⁺ or HLA-A2⁻ dexosomes at the peptide concentrations of 0.01 to 10 μ g/ml. Loaded dexosomes were subsequently washed by density gradient centrifugation to remove unbound peptide. The results are presented Figure 24 and show that HLA A2⁺ BM-DC cells pulsed with 10 μ M of Mart were able to activate LT11 clone that recognized HLA A2 with the Mart peptide. The HLA A2⁻ BM-DC cells can't stimulate LT11 clone.

BM-DC cells pulsed with 15 μ l of Mart directly loaded HLA A2⁺ dexosomes were able to activate LT11 clone whatever the haplotype of the dendritic cells.

Previously, indirect peptide loaded dexosomes only induced marginal levels of IL-2 secretion even when 10 μ g/ml Mart-1 peptide (SEQ ID NO. 3) was added to the DC culture to prepare the dexosome. Dexosomes directly loaded with Mart-1 peptide (SEQ ID NO. 3) consistently and reproducibly stimulated the LT 11 clone, suggesting that direct peptide loading is a more efficient way to produce biologically active dexosomes.

The same protocol was applied for direct loading of an HLA-A1 restricted peptide. In the experiment, a biotinylated reference peptide EVDPC(biotin)GHLY, derived from the natural HLA-A1 restricted, MAGE3-A1 peptide EVDPIGHLY, (SEQ ID NO. 2) binds to A1⁺/A2⁻ dexosome (Exo

433), but not to A1⁻/A2⁺ dextran (Exo 427). Conversely, the HLA-A2 restricted, biotinylated reference peptide derived from Hepatitis B core antigen, (SEQ ID NO. 1) binds to A1⁻/A2⁺ but not to A1⁺/A2⁻ dextran, demonstrating a strict HLA-allele-dependent loading of Class I antigen peptides. The bottom panel of Figure 10 shows unlabeled MAGE3-A1 peptide (SEQ ID NO. 2) competed with the biotinylated MAGE-3A1 peptide (SEQ ID NO. 2) for binding on A1⁺ dextran (Exo 433).

The direct loading approach was also applied to a PIA peptide and murine dexosomes. The results are presented Figure 25. D1 cells pulsed with 25 µl or 50 µl of OVA (SEQ ID NO. 4) directly loaded H2^b dexosomes were able to activate B3Z cells that recognizes K^b with the OVA peptide (SEQ ID NO. 4). We also observed, to a lower extent, an activation when D1 cells were pulsed with OVA directly loaded H2^d dexosomes.

On page 90, line 18 through page 92, line 6, please replace with the following paragraphs:

Based on the above results, we loaded Mart-1 peptide (SEQ ID NO. 3) to HLA-A2⁺ in the absence of α 2-m and tested them in LT 11 assay. Figure 30 shows biological activity of dexosomes loaded with Mart-1 peptide in the absence of α 2-m. In this experiment, Mart-1 peptide (SEQ ID NO. 3) at 10 and 100 µg/ml was loaded onto HLA-A2⁺ (Exo 447) and HLA-A2⁻ (Exo 450) dexosomes at both pH 4.8 and 5.2 in the absence of α 2-m. As control, Exo 447 was also loaded with 10 µg/ml of Mart-1 in the presence of α 2-m. Dexosomes loaded at the three different conditions (pH 4.8 without α 2-m, pH 5.2 without α 2-m, pH 4.2 with α 2-m) all had similar biological activity, indicating that α 2-m is not absolutely required for generating biologically active dexosomes.

Direct loading of HLA-A1⁺/B35⁺ dexosome in the presence and absence of α 2-m

The same protocol can be applied for direct loading of an HLA-A1 or B35 restricted peptide. The nonameric MAGE-3A1 (EVDPIGHLY), (SEQ ID NO. 2) derived from MAGE-3 protein is presented by HLA-A1 and HLA-B35. To test whether this peptide can be loaded on HLA-A1⁺ dexosomes, we designed a reference peptide named MAGE-3A1C5 (SEQ ID NO. 5) in which the isoleucine of the MAGE-3A1 peptide is substituted with a cysteine and labeled by biotin. In the experiment shown in top panel of Figure 31, the biotinylated MAGE-3A1C5 (SEQ ID NO. 5) was loaded to a HLA-A1⁺/A2⁻ dexosome (Exo 433) in the presence of β 2-m. We found the same peptide could not be loaded to HLA-A1⁻/A2⁺ dexosome (Exo 427). Conversely, the HLA-A2 restricted, biotinylated reference peptide derived from Hepatitis B core antigen (SEQ ID NO. 1), could be loaded to A1⁻/A2⁺ but not to A1⁺/A2⁻ dexosome, demonstrating a strict HLA-allele-dependent loading of Class I antigen peptides. The bottom panel of Figure 31 shows the natural MAGE3-A1 peptide (SEQ ID NO. 2) competed with the biotinylated MAGE-3A1C5 peptide (SEQ ID NO. 5) for binding on A1⁺ dexosome (Exo 433), indicating the binding capacity of unlabeled MAGE3-A1 peptide to the A1⁺ dexosome

Because of limited source of HLA-A1⁺ dexosome, we used HLA-B35⁺ dexosome (Exo 426) to test the binding of MAGE 3-A1 (SEQ ID NO. 2) and inhibition in the absence of β 2-m. Using the same loading conditions for HLA-A2⁺ dexosome, we directly show in the top panel of Figure 32 that MAGE-3A1C5 (SEQ ID NO. 5) can bind to HLA-B35⁺ dexosome at either pH 4.8 or pH 5.2, with less non-specific binding to HLA-A1⁻B35⁻ dexosome at pH5.2 (Exo 424). The bottom panel of Figure 32 shows the inhibition of MAGE-3A1C5 (SEQ ID NO. 5) by natural MAGE-3A1 (SEQ ID NO. 2) peptide at pH 4.8 and 5.2 in the absence of β 2-m. Binding inhibition by natural MAGE-3A1 was much stronger at pH 4.8, suggesting better binding capacity of the unlabeled MAGE-3A1 peptide (SEQ ID

NO. 2) to HLA-B35⁺ dexosome at this pH. These results show the broad application of direct peptide loading on other HLA Class I alleles.

On page 93, lines 9-18, please replace with the following paragraph:

Direct loading can also be done in the absence of β 2-m. Using a high peptide concentration and a mild acid buffer, peptide exchange on HLA molecules was observed. We have demonstrated by competition experiment that our target peptides MAGE-3, 4, and 10 can be specifically loaded to HLA-A2⁺ dexosomes in this manner. Mart-1 peptide (SEQ ID NO. 3) loaded dexosome in the absence of β 2-m stimulated IFN- γ secretion by LT 11 demonstrating the biological activity of the dexosome. Therefore manufacturing biologically active dexosome without β 2-m is feasible thus eliminating the present difficulties of using β 2-m purified from human donors or non-GMP grade recombinant β 2-m. The overall loading is lower in the absence of β 2-m, but it does not seem to affect LT 11 activation, suggesting the bioassay is much more sensitive than biochemical assay.

These amendments are made in response to the (Second) formalities letter from the Patent & Trademark Office. A statement under 37 CFR § 1.821 is filed herewith.

The Applicant's attorney of record hereby authorizes the Commissioner to charge a two-month extension fee of \$450.00 to Orrick, Herrington & Sutcliffe's Deposit Account No. **150665** and to credit any overpayments to said Deposit Account No. **150665**.

Respectfully submitted,

ORRICK, HERRINGTON & SUTCLIFFE LLP

Dated: April 21, 2005

By: 

Kurt T. Mulville
Reg. No. 37,194

4 Park Plaza, Suite 1600
Irvine, CA 92614
949/567-6700 Telephone
949/567-6710 Facsimile